REMARKS

The front page of Advisory Action indicated a mailing date of April 24, 2006. Applicant did not receive such Advisory Action until May 9, 2006, a delay of some fifteen (15) days after the aforesaid mailing date. Both dates extend beyond the six-month period from the date of the Final Rejection of October 18, 2005 (hereinafter "Final") i.e., April 20, 2006. Such untimely response required Applicant to file a Notice of Appeal to prevent the instant application from becoming abandoned on April 20, 2006, prior to the indicated April 24, 2006 mailing date of the Advisory Action. Applicant filed a Notice of Appeal on April 18, 2006 to avoid such abandonment, even though the status of the claims was not timely communicated by the Examiner.

Applicant believes that he has been prejudiced because the Patent and Trademark Office (hereinafter "PTO") clearly did not handle the instant application in a timely manner consistent with its rules and guidelines. This situation is the subject of a Petition to the Group Director of Group 1600 and need not be discussed further in this paper. In any event, the Examiner is again requested to treat the instant application in accordance with the guidelines established for cases having Special Status; otherwise, the term for any subsequent patent will continue to be needlessly eroded.

Turning to the rejection, claims 383, 384, 391, 393, and 394 remain rejected under 35 U.S.C. §112, second paragraph; and claims 382-394 remain rejected under U.S.C. §102(b) as being anticipated by Lutjen et al. (hereinafter "Lutjen").

Applicant submits the following response to the rejection of claims 383, 384, 391, 393, and 394 under 35 U.S.C. §112, second paragraph, as being indefinite.

In the Advisory Action, the Examiner stated that the basis of this rejection is "for reasons of record" without specifically identifying such basis. Applicant believes that the prosecution of the instant application would be materially advanced and compacted if the Examiner provided a clear statement of the rejection rather than merely state that such rejection is "for reasons of record." Otherwise, the basis remains obscured because it is unclear as to which of the numerous, varied grounds previously relied upon by the Examiner continue to be remain relied upon, are withdrawn, or are modified by the Examiner. Should any rejections be maintained, following consideration of this paper, the Examiner is requested to set forth the specific basis for such rejections in any subsequent Office Action and not merely state that such rejections are for reasons of record.

It appears from the Advisory Action that the Examiner failed to consider and make of record the Sixth Supplemental Information Disclosure Statement filed February 21, 2006, via fax. Accordingly, the Examiner is requested to indicate that such document is of record and to fully consider the references cited therein and the comments regarding such references.

The rejection of claims 383, 384, and 394 is understood to be on the basis that the term "multifactorial and non-specific" is indefinite. Applicant disagrees that said claims fail to satisfy the "definiteness" requirement of the statute and proffers the following remarks and evidence in rebuttal.

Applicant is confounded as to why this ground of the rejection was made and maintained (for reasons that have shifted during the course of this prosecution) because such rejection is not consistent with a prior PTO decision. In the Sixth Supplemental Information Disclosure Statement, Applicant called the Examiner's attention to the fact that parent application Serial

Number 08/326,857 was granted to Applicant on June 2, 1998 as Patent Number 5,759,033 (hereinafter referred to as "the '033 patent"). The Examiner failed to respond to such submission, and thus the Examiner's attention is again directed to the '033 patent. Claims 13 and 26 of this patent specify that the claimed growth factor is multifactorial and non-specific. The grant of these claims by the PTO constitutes compelling evidence that the definiteness requirements of 35 U.S.C. §112, second paragraph, were satisfied and that both disputed terms were understood. Applicant submits that the current Examiner's rejection involving such previously accepted terminology is prejudicial to the above-mentioned claims of the patent and has the potential to raise validity issues of the issued patent. Such action amounts to a failure to accord full faith and credit to the previous action of the PTO. See MPEP Section 706.04. Unless the Examiner can provide compelling evidence that the allowance of such claims was erroneous, the earlier action must be maintained. Applicant believes that no such compelling evidence has been presented by the Examiner. To the contrary, Applicant believes that the following remarks and evidence fully demonstrate that the disputed terms are definite and understood by one skilled in the medical art, and thus the prior action of the PTO remains correct and controlling.

Independent evidence supporting the prior PTO decision may be found in the results of Applicant's search, using the Google search engine, for the term "multifactorial growth factor." Several publications using the questioned term to describe growth factors were located thereby. In this regard, J. Biol. Chem, Vol. 280, August 5, 2005 (attached hereto as Exhibit A) relates to using the integrative nuclear fibroblast multifactorial growth factor FGFR 1. Furthermore, J. Eukaryot Microbiol, 49(5), 2002 pages 383-390 (attached hereto as Exhibit B) discloses that epidermal growth factor (EGF) is a multifactorial growth factor that activates signal transduction

events in mammalian cells. Both fibroblast (FGF) and epidermal (EGF) growth factors are described as multifactorial growth factors capable of promoting the growth of soft tissue in the body of the patient on pages 20 and 21 of the specification.

Applicant is aware that a portion of the Examiner's position involves whether or not cells can be described to be multifactorial. In as much as the Examiner already has acknowledged that cells are growth factors, Applicant can find no apparent reason why the Examiner should continue to assert that cellular growth factors, somehow, could not be described as multifactorial in view of the publications cited in the previous paragraph.

Applicant directs the Examiner's attention to the recent *en banc* decision of the CAFC in Phillips v. AWH Corporation, 03-1269-1286, decided July 12, 2005. While the Phillips case involved patent claim infringement, Applicant believes that the principles and authorities expressed in this case are equally applicable for providing guidance to the PTO in determining the meaning of terms in the specification and claims of a pending patent application.

The Phillips decision indicated that the claims of a patent are generally given their ordinary and customary meaning in the art, citing the Vitronics v. Conceptronic, Inc., 90 F. 3d 1582 (Fed. Cir. 1996). Also cited was the Multiform Desiccants, Inc. v. Medzorn, Ltd. Decision, 133 F. 3d 1473, 1477 (Fed. Cir. 1980) for the principle that claims should be read in the context of the patent. The Court in Phillips further observed that extrinsic evidence is less significant than the intrinsic record in determining the legally operative meaning of claim language, citing C.R. Bard, Inc. v. U.S. Surgical Corp., 388 F. 3d 858, 862 (Fed. Cir. 2000). The Court in Phillips also stated that dictionary evidence can be useful in claim interpretation, but that such evidence is less reliable than the patent specification and its prosecution history. Applicant

submits that the Examiner should interpret the words "multifactorial" and "non-specific" in light of the specification as would be apparent to a person skilled in the medical art and thus give such words their ordinary meaning in the art to which the invention pertains. A different interpretation, such as that foisted by the Examiner, bottomed on non-contextual sources, places the term out of context and thus clearly would not be entitled to the same evidentiary weight as the interpretation by a skilled person in the medical art of Applicant's disclosure.

In the instant prosecution, the Examiner attempts to support her reasoning by a lack of success in regard to search results for the term "multifactorial and nonspecific cell", followed by a series of suppositions and speculations regarding the meaning of these terms. As demonstrated above, the Examiner' failed search appears to have been improperly conducted and certainly does not support the Examiner's position. Thus, Applicant believes that the Examiner's position amounts to no more than opinion because no meaningful objective evidence related to the medical art is presented. Had the Examiner viewed the terms "factor", "multifactorial", and "non-specific", as understood by a skilled medical person, she would not have raised an issue of indefiniteness. The meaning of the term "factor" is well known in the medical art, and one skilled in such art would have no difficulty understanding this term. Obviously, anyone understanding the medical term "factor" would also understand the term "multifactorial" to mean "more than one factor."

The Examiner is reminded that Applicant previously located and filed relevant search evidence in the Fifth Supplemental Information Disclosure Statement ("IDS") filed on October 21, 2004, via fax, regarding the definitions of the questioned terms. The definitions of "multifactorial" and "non-specific" presented in the IDS provide confirming evidence that the

disputed terms are known and used properly in Applicant's specification. Note further that the IDS identifies these term as adjectives.

Applicant also reminds the Examiner that a search of the NIH Medical Dictionary was conducted by Applicant and is of record. The following definitions in Merriam Webster's Medline Plus Medical Dictionary were found:

Factor:	***(noun)*** A substance that functions in or promotes the function of a particular physiological process or bodily system.
Multifactorial:	***(adjective)*** Having, involving, or produced by a variety of elements or causes.

Thus, the noun "factor," as used in Applicant's specification, means a substance, such as a cell, that promotes a particular physiological process, such as the formation of a bud and subsequent growth of soft tissue. "Multifactorial" is an adjective used to denote a quality of a cell. A cell is deemed to be "multifactorial" when a variety (more than one) of elements (factors) promote the growth of soft tissue. Accordingly, there can be no doubt that the term "multifactorial" is used properly in the specification, and that its meaning would be clear to one skilled in the medical art. The above-mentioned definitions are consistent with Applicant's specification; with the materials furnished in the IDS; with the use of this term by those skilled in the medical art, such as Drs. Heuser and Lorincz; and with the previous determination of the PTO.

As mentioned in the preceding paragraph, the questioned terms were "read and understood" by skilled persons in the art, i.e., by Dr. Heuser in his Declaration (of record) and in

his concurrently-submitted Second Supplemental Declaration (attached hereto as Exhibit C) and by Dr. Lorincz in his Supplemental Declaration (of record) and in his concurrently-submitted Second Supplemental Declaration (attached hereto as Exhibit D). The Examiner criticized such evidence, as it pertained to Dr. Heuser's Declaration and Dr. Lorincz' Supplemental Declaration, in the Advisory Action because "they do not explain what cells are encompassed by the term." Such criticism misses the point because it is clear that the terms were read and obviously understood by these two experts in the medical art, thereby showing that the terms are, in fact, definite. It is noted that all of the above-mentioned declarations state that relevant portions of the specification regarding multifactorial and non-specific cells were "read and understood" by Drs. Heuser and Lorincz, thereby further underscoring that such terms are understood by those skilled in the medical art.

The Examiner is reminded Applicant's specification indicates that multifactorial and non-specific cells may include stem cells and germinal cells. The Examiner has concocted an issue that other cells are not mentioned. Initially, the Examiner erroneously raised the issue that only stem cells were included. Upon Applicant pointing out that germinal cells were also included in the specification, the Examiner then changed the issue to what further cell types were included. Applicant believes that the disclosure of the above-mentioned two types of multifactorial and non-specific cells, along with pluripotent and bone marrow stem cells, is fully adequate to describe examples of types of cells having the described characteristics.

Another example of the definiteness of the questioned terms by workers skilled in the medical art consistent with the description in Applicant's specification, the definition of "multifactorial" in the IDS, the above-mentioned NIH Medical Dictionary, and Drs. Heuser and

Lorincz can be found in Strauer 2005 (of record). Dr. Strauer states at page 1656, second column, third paragraph that, "The regenerative potential of bone-marrow-derived stem cells may be explained by any of four mechanisms." These four-cell biologic and molecular mechanisms are further described as "factors" at page 1657, second column, second full paragraph. Therefore, it is clear to a skilled person in the medical art that Dr. Strauer and his co-authors identify the regenerative potential of bone marrow stem cells as being derived from at least four different mechanisms/factors or characteristics of such cells. It follows that bone marrow stem cells can be appropriately styled as four-factor cells, i.e., multifactorial. Thus, Strauer 2005 confirms that yet another skilled group of medical experts possesses an understanding of "multifactorial" cells that is consistent with that of Applicant, the evidentiary materials discussed herein, and the PTO.

In the Advisory Action, the Examiner considered that, "Even if Strauer 2005 could be tortuously construed as describing bone marrow stem cells as multifactorial, Strauer 2005 only discusses bone marrow stem cells." Note further that Applicant described using the same stem cells as Strauer 2005, bone marrow stem cells. Whether acknowledged as tortuous or being clearly evident to one skilled in the medical art, the fact remains that Strauer 2005 fairly and reasonably teaches that a type of stem cells are multifactorial and thus supports Applicant's position. It would seem to Applicant that the above quoted passage of the Examiner should serve to conclusively resolve this issue.

The Examiner's attention is again directed to yet another publication in which a skilled medical person utilizes the term "multifactorial" in a manner consistent with Applicant's specification; namely, the 2001 publication of Caplan et al. (attached hereto as Exhibit E and

hereinafter "Caplan 2001") entitled, "Mesenchymal stem cells: building blocks for molecular medicine in the 21st century." Note the use of the term "multifactorial" in this publication. Caplan 2001 teaches that mesenchymal stem cells prevalent in bone marrow are pluripotent in that they are capable of differentiating into multiple tissues types. Caplan 2001 further teaches that such bone marrow stem cells undergo multifactorial differentiation pathway from stem cells to functional tissues including elaborate composite tissues *in situ*. This description is consistent with Applicant's use of the terms "multifactorial" and "non-specific" to define pluripotent cells such as bone marrow stem cells and germinal cells, which induce or promote the growth of composite soft tissues.

In the Sixth Supplemental Information Disclosure Statement, Applicant also submitted an earlier publication of Caplan, namely, a publication entitled "Mesenchymal Stem Cells," Journal of Orthopaedic Research, Volume 9, No. 5, 1991, pages 641-650 (hereinafter "Caplan 1991"). Caplan 1991 describes bone marrow stem cells as exhibiting multifactorial characteristics. Accordingly, Applicant believes that Caplan 1991, like Caplan 2001, contains compelling evidence that those skilled in the medical art understand and use the questioned terms in a manner consistent with Applicant's use thereof. Caplan 2001 described mesenchymal stem cells ("MSC"), which were harvested from bone marrow and/or periosteum, as comprising multifactorial cells. Specific passages of Caplan 2001 are referenced below in support of Applicant's position.

Regarding Applicant's use of the term "multifactorial cells," which cells are species of the described and claimed genus "growth factor," Caplan 1991 recognized and attributed

multifactorial characteristics to MSC at page 641, left column paragraph 1, lines 8-14 and at the top of page summary, lines 6-8.

The first reference at page 641 is as follows:

> Their progeny are affected by a number of factors, however, as they become tracked into very specific developmental pathways in which both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire (9,11).

The second reference at page 641 is as follows:

> Local cuing (extrinsic factors) and the genomic potential (intrinsic factors) interact at each lineage step to control the rate and characteristic phenotype of the cells of the emerging tissue.

As should be understood by the Examiner, Caplan 1991 clearly characterizes MSCs as multifactorial cells.

Applicant's search also revealed the Merck Manual of Geriatrics, Ch. 72, Cancer (attached hereto as Exhibit F), which describes "Oprelvekin, a nonspecific growth factor for megakaryocytes" and the NIH Pub Med abstract identifying "Erythropoietin as a nonspecific growth factor and its effect on carcionogenesis" (attached hereto as Exhibit G). Regarding the limitation "nonspecific" cells, species of the genus "growth factor", Caplan 1991 disclosed that MSCs are lineage-nonspecific, i.e., they can develop into nine (9) separate and unique tissues. In this regard, see Fig.1. page 642. Thus, it is patently clear that the art skilled understand the meaning of the term "nonspecific" when applied to cells such as stem cells - they are lineage-nonspecific and can develop into a variety of tissues. For the Examiner to deny this fact requires a denial of pure science.

It is submitted that Applicant's above-mentioned evidence, when considered with the authoritative statements and precedential tenets of Phillips, must be accorded far greater evidentiary weight than the Examiner's unsubstantiated speculation as to the intended meaning of the questioned term. When following the Phillips decision and thus reading the claim language within the context of the specification with the understanding of a person skilled in the medical art, Applicant believes that there can be no question as to the meaning of "multifactorial." The meaning of "non-specific" as being synonymous with "non-specialized" is apparent from previous submissions and the above-mentioned evidence.

When it is considered that the PTO previously determined that the questioned terms are definite, that Drs. Heuser and Lorincz read and understood such terminology, and that Applicant presented a large body of independent evidence supporting, and consistent with, the prior determination of the PTO and Applicant's medical experts, it appears that the Examiner is the only one that does not understand such terms. Surely, the Examiner's lack of understanding cannot overcome such compelling evidence. Accordingly, this aspect of the indefiniteness rejection should be withdrawn.

Applicant submits that the Examiner also erred in concluding that claims 383 and 384 are contradictory. Claim 383 requires "multifactorial and non-specific cells." Claim 384 further limits claim 383 by reciting that the cells are "stem cells." The specification at page 37 describes stem cells and germinal cells as included in the class multifactorial and non-specific, and on page 50 further describes multifactorial and non-specific growth factors, such as the "stem cells and germinal cells" described on page 37, as being "pluripotent." Accordingly, it requires no more than a basic understanding of patent claim construction to conclude that claim 384 is in full

compliance with the fourth paragraph of Section 112. Further, it is noted that the Examiner's statement on page 10 of the Final that, "The specification states, "Multifactorial and nonspecific cells (such as stem cells...) ..." Such implies that all stem cells are multifactorial and nonspecific, in direct contradiction to Applicant's arguments." This statement is simply not correct. The Examiner appears to have not fully quoted Applicant's specification. As can be evinced from the complete disclosure on page 37 of the specification, Applicant describes "stem cells and germinal cells" (emphasis added) as being exemplary of "multifactorial and non-specific cells." Claim 394 is deemed proper since it further limits claims 393 and 391, from which it directly and indirectly depends. Such limitation is proper because claim 391 broadly includes both single factor and multifactor cells, and dependent claim 393 further limits such cells to multifactorial cells. The specification on page 48 clearly discloses that, "germinal cells (and in some cases, stem cells,)" can result in growth and differentiation of an organ. This is a clear teaching that not all stem cells result in morphogenesis of an organ. Thusly, Applicant's specification clearly discloses that, to one skilled in the medical art, while multifactorial and non-specific cells are stem cells, not all stem cells are multifactorial and non-specific.

In summary, Applicant believes that once the Examiner's misunderstanding of the questioned terminology is transformed into an understanding consistent with that used by a skilled person in the medical art, there should be no further indefiniteness question remaining. Applicant hereby repeats the remarks (and associated evidence) that were presented in the August 2, 2005 Response. These remarks are not incorporated into the instant response so as to not burden the record; however, such remarks are consistent with the above remarks and are maintained and deemed to be persuasive. Applicant submits for all of the above reasons that

claims 383, 384, 391 and 394 are in compliance with the definiteness requirement of the statute and that the Examiner's rejection should be withdrawn.

Claims 382-394 stand rejected under 35 U.S.C. §102(b) as anticipated by Lutjen "for reasons of record." Applicant disagrees that Lutjen constitutes anticipatory prior art in regard to new claims 382-402, within the purview of Section 102. As revealed earlier, claim 382 has been amended to recite that the grown and integrated tissue consists "of a desired soft tissue ... which integrates itself into said body of said human patient." New claim 395 further limits the subject matter of claim 382 by reciting that the "cells are injected" into the patient's body. New claims 396-399 further limit the subject matter of claim 382 by requiring local placement of the cells e.g., where the cells are "injected intramuscularly" (claims 398, 399). New claims 400 and 401 add the step of confirming new artery growth. New claim 402 recites that the selected site is a leg of the patient. Applicant submits that Lutjen does not respond to the subject matter defined in claims 382-402. Favorable reconsideration of this rejection is requested in view of the following remarks.

The language of claim 382, as amended, precludes Lutjen's in vitro fertilization implantation by limiting the claimed method to growth of tissue "consisting of a desired soft tissue." Lutjen clearly does not respond to such defined method. Neither does Lutjen respond to placing "stem cells" or "pluripotent cells" in the body of a human patient to grow tissue "consisting of a desired soft tissue" (claims 384, 389, 391, 393, 397); forming soft tissue consisting of a new artery "which integrates itself " in the patient's body (claims 385, 386, 388, 390). Nor does Lutjen respond to a method where cells are injected, such as, intramuscularly or where the selected site of growth is a leg of the patient (claims 395, 398, 399, 402).

The Examiner's argument at pages 10 and 11 of the Advisory Action that, "cells used by Lutjen clearly integrate in the body of the human patient in that all human pregnancies proceed via invasive implantation wherein the maternal cells completely envelop the fetus" is inapt. The Examiner's equating of the term "integrate" with "envelope" on page 8 of the Advisory Action apparently forms the basis for such determination.

As pointed out above, the Examiner's position is bottomed on an incorrect interpretation of the claimed limitation of "integrating" as used and intended by Applicant in describing the claimed invention. Following the precedent of the Philips decision, it is incumbent upon the Examiner to read the claim limitations in the context intended in the specification. It is patently clear from Applicant's specification that the grown soft tissue is permanently integrated into the patient's body. See, in particular, page 54 wherein it is disclosed that a new artery is grown adjacent the patient's original artery and "has integrated itself" with the original artery and page 56 wherein the newly grown artery is disclosed to "integrate itself in the heart." Also see page 45 wherein is disclosed that an artery can be grown in the heart, legs, or other areas by injecting genetic material (cells) into muscle at a desired site, and that a damaged portion of a heart can be used as a matrix while the new muscles and vessels grow. Also see the disclosure at page 46 where it is disclosed that the newly grown muscle may integrate itself into the existing muscle. It is clear from such disclosure that the limitation "integrate" as used in the specification and claims at issue defines permanently integrating the grown soft tissue into the patient's body. This is the precise contribution that Applicant has made to the therapeutic medical art. This is the definition and meaning that one skilled in the medical art reading the specification and claims, in proper context, would give to the "integrating" language of the claims. See Philips, supra.

The Examiner's attention is directed to the concurrently submitted Second Supplemental Declarations of Drs. Heuser and Lorincz wherein these skilled medical experts, upon reading and understanding applicable portions of Applicant's specification, determined that the grown soft tissue, such as an artery, "will integrate itself into pre-existing tissue of the body thereby forming a unified whole." Such understandings constitute compelling evidence that those skilled in the medical art would understand the term "integrate" differently than the Examiner's understanding of the term.

It is true that the term "integrate" – whether employed in a medical context or in common everyday use – is a plain English word which is readily defined in competent dictionaries. The term "integrate" as used in Applicant's specification and claims is consistent with the definition provided by such dictionaries. Exemplary of such are Medline Plus, Merriam-Webster Medical Dictionary, 2005, which defines integrate as *"to form or blend into a unified whole: cause to undergo integration"* (attached hereto as Exhibit H) and Merriam-Webster's Collegiate Dictionary, 10th Ed., 1993 which defines integrate as *"to form, coordinate, or blend into a functioning or unified whole: unite"* (attached hereto as Exhibit I). Neither dictionary defines "integrate" to mean "envelop." Nor has the Examiner provided evidence in the form of a dictionary definition equating integrating with enveloping. If the Examiner is aware of a medical dictionary, or even a standard "plain English" dictionary that supports her position, it behooves her to enter such evidence in the record for critical review rather than to provide her own, unsupported interpretation of such term. This is yet again another instance where the Examiner has foisted her own interpretation of the meaning of language and terms in Applicant's specification and claims in an illogical manner that is patently inconsistent with their intended

meaning. The specification does not simply imply that an artery is permanently integrated; nor has Applicant implied thusly. Rather, the specification is replete with explicit statements that the soft tissue (artery) integrates itself in the patient's body i.e., blends into/unites with the patient's body. It would be clear to the reader of the specification and claims that Applicant's invention involved the growth and permanent integration of soft tissue, more specifically an artery, into a desired site in the patient's body. It is further highly illogical for the Examiner to equate a developing fetus "enveloped" in the mother's womb to the body of the patient defined in the specification and claims in issue. Even an unskilled person would readily recognize that the affect of uniting (integrating) a placenta with the host mother can only result in a catastrophic event. While it is proper in determining patentability to read claim language broadly, claims must be interpreted in light of the specification and in view of the background of one skilled in the pertinent art. It is never proper to employ illogical, bogus, and unsupported reasoning in making patentability determinations.

When viewing the claims in the above light, it is patently clear that an unborn fetus is not integrated into the patient's body. Stedman's Medical Dictionary, relied upon by the Examiner for a definition of "placenta," provides evidence that a fetus is not integrated into the mother's body by its statement that there is "no direct mixing of fetal and maternal blood, but the intervening tissue (the placental membrane) is sufficiently thin to permit the adsorption of nutritive materials..." The Examiner's reference on page 8 of the Advisory Action to the placenta comprising an organ of metabolic exchange between the fetus and the mother comprised of a fetal portion i.e., the outer extra-embryonic membrane (chorion frondosum) and a host portion i.e., the indemetrium (decidua basalis) as somehow meeting the claimed requirement

that soft tissue integrates itself in the patient's body, misses the point because this organ is shed at parturition. Because the placenta is shed at childbirth it cannot be considered to be integrated – and thus form a uniform whole – with the mother host. Further, Stedman's defines "percreta" as a condition that occurs when the placenta does integrate with the uterus resulting in a catastrophic rupture of the uterus. An ectopic pregnancy is another well-known example of a medical condition where the fetus integrates with the mother and results in a catastrophic event. Both of these events result in termination of the fetus. It is patently clear from such evidence that the Examiner's assertion (Final, page 21) that, "a fetus, and all of its parts, are integrated into to [sic] body of a human patient" lacks sound factual basis. Should any such harmful integration have occurred in Lutjen, it would not have been possible for the baby to be born. Thus, Lutjen did not "integrate" the fetus into the woman. Perforce, it cannot reasonably be said that Lutjen responds to growing soft tissue which "integrates itself " in the patient's body as called for by the claims.

In any event, claim 382, as amended, excludes the growth of hard tissues by requiring that the grown tissue "consists of soft tissue". The "consisting of" language closes the claim to cells that promote the growth of hard tissue. Thus, Lutjen's totipotent two-cell embryo is excluded and, therefore, does not respond to the cell required in claim 382. While the question as to whether a totipotent two-cell embryo is a stem cell is academic in regard to the amended claims, there can be no doubt that the NIH believes that while a pluripotent stem cell "can form virtually any type of cell found in the human body, they cannot form an organism because they are unable to give rise to the placenta and supporting tissues necessary for development in the human uterus...". Because their potential is not total, they are not totipotent and they are not

embryos. In fact, if an inner cell mass was placed in a woman's uterus, it would not develop into a fetus." See the NIH publication entitled, "Stem Cells: A Primer," pages 1 and 2 (of record in Applicant's response filed December 2, 2005). Such scientific evidence authored by the NIH is totally consistent with Applicant's specification, claims, and arguments.

The Examiner's statements, at pages 3 and 4 of the Final, that all pluripotent cells are stem cells, and that all stem cells are at least pluripotent were not repeated in the Advisory Action and have apparently been conceded to be incorrect in view of the authorities (evidence) and supporting arguments presented in Applicant's December 2, 2005 response. Further, in this regard, see the specification at page 48, lines 13 and 14 where it is disclosed that germinal cells (and in some cases, stem cells) can be used to form an organ through differentiation and morphogenesis. This is a clear disclosure of the concept that not all stem cells are pluripotent. A similar concession is apparently true for the statement in the Final that, "There is no such thing as a unipotent stem cell," since it was not repeated in the Advisory Action.

Applicant believes that the Examiner's statement that none of the claims exclude totipotent cells is misplaced. The scope of a claim is determined by the limitations expressly contained therein. Further, the Examiner's statement that claims 382, 385, 387 and 388 do not place limitations on the cells is lacking in merit because in each instance the claims require a class of cells that induce the growth of soft tissue, which integrates with the patient's body. If particular cells, for example, as in the case of Lutjen, are totipotent, such cells are incapable of growing and integrating an artery in the patient's body (claims 385, 388) and are therefore excluded from the claims. The Examiner's attempt to hold the claim language indefinite and at the same time consider the subject matter defined by such language to reasonably read on

Lutjen's two-cell embryo is derogating to the record and current law.

The Examiner's statement that totipotent cells are stem cells is not correct. Lutjen does not use the term "stem cell" to describe the two-cell embryo. Thus, it is the Examiner, not Lutjen, that has so characterized the two-cell embryo. The above-mentioned NIH publication entitled, "Stem Cells: A Primer," defines stem cells as "cells that have the ability to divide for indefinite periods in culture and to give rise to specialized cells." While Lutjen's two-cell embryo is a totipotent cell – meaning that its potential is total in that it can give rise to an entire human organism – it is not a stem cell because it does not have the ability to self-renew *in vitro* virtually indefinitely. This definition is consistent with the definition for "stem cells" contained in the article published by the University of Pittsburgh Medical Center on bio.com entitled, "Discarded Placentas Deliver Researchers Promising Cells Similar to Embryonic Stem Cells" (of record in Applicant's response filed December 2, 2005). Such totipotent cell specializes to form a blastocyst the inner cell mass of which specializes into pluripotent cells. Pluripotent cells are not totipotent because their potential is not total, and they are not embryos. In addition, pluripotent cells do not form a placenta. This publication makes it clear that if an inner cell mass cell, i.e., an embryonic stem cell, were placed in a woman's uterus; it would not develop into a fetus. It is again emphasized that while it is clear to those skilled in the medical art that pluripotent cells are incapable of forming a placenta, pluripotent cells can be harvested from placenta tissue. The Examiner has not provided any evidence to support her position that an embryonic stem cell is considered to be totipotent in the medical art. Thus, it is patently clear that the two-cell embryo employed by Lutjen does not respond to the cell required in Applicant's claims for "growing and integrating" desired soft tissue in the patient's body. This is especially

true for the "multifactorial and non-specific" cell required by claim 383, the stem cells required by claims 384, 389, and 393, and the "pluripotent cells" required by claim 391. Applicant's specification and claims do not broadly describe using an embryo, and Applicant's disclosed invention would not be operable using such a cell. Nor would the implantation of an embryonic stem cell in the uterus of a female produce the results described by Lutjen. The Examiner's statement that the claims do not require that the implanted cells be unable to form a placenta is considered to be inapt. The claims, by reciting "growing and integrating tissue consisting of a desired soft tissue...which integrates itself into" a human patient's body, preclude the formation of a placenta and fetus. Accordingly, it cannot be reasonably concluded that the two-cell embryo used by Lutjen responds to the cellular growth factors required in Applicant's claims. It is patently clear that the claimed subject matter requires a different implant material and a claimed result that is distinct from that described by Lutjen.

Applicant hereby repeats the remarks and relies upon the associated evidence that was presented in the August 2, 2005 response. These remarks are not incorporated into the instant response so as to not burden the record; however, such remarks are consistent with the above remarks and are maintained and deemed to be persuasive.

Although Applicant believes that the Section 102 rejection should be withdrawn for the reasons set forth above, the following additional points raised by the Examiner in the Final will be addressed in a sincere attempt to clarify the record. The Examiner has mischaracterized the cited Fukuda et al. (hereinafter "Fukuda") reference as being exemplary of the prior art's considering embryonic stem ("ES") cells to be totipotent in an attempt to support her statement that ES cells are totipotent (Final, page 16). Both are incorrect. Fukuda teaches that, "ES cells

are derived from the inner mass of the preimplantation blastocyst" (page 1273) and ES cells are pluripotent (page 1275). Fukuda at page 1273 does teach that ES cells have the ability to differentiate into any cell type of an organ. This defines a pluripotent cell, not a totipotent cell. The PTO should take Official Notice of the fact that pluripotent cells can make virtually any cell type of an organ but cannot make so called "extra embryonic" tissues such as the amnion, choroin and other components of the placenta. For the Examiner to contend otherwise is simply astonishing and evinces a lack of clear understanding in regard to cell biology. Further, Fukuda teaches that ES cell lines require the destruction of embryos (page 1278). Fukuda also teaches that adult bone marrow is pluripotent (page 1278). Thus, rather than support the Examiner's position, Fukuda is evidence of the correctness of Applicant's specification in regard to the disclosed and claimed cellular growth factors.

The Examiner's reliance on Satoh et al. (hereinafter "Satoh") is still not understood. Applicant has never said that any and all pluripotent cells are capable of forming all three types of soft tissues. Satoh teaches that pluripotent hematopoietic stem cells are defined as cells capable of both self-renewal (i.e., stem cells) and multilineage (non-specific lineage) differentiation (i.e., pluripotency). This clearly corresponds to Applicant's statement that pluripotent cells can promote the growth of all three major soft tissue types i.e., are capable of multilineage differentiation. Is it the Examiner's contention that no pluripotent cell is capable of forming all three types of soft tissues? How does this square with the Examiner's reliance on Fukuda on page 11 of the Advisory Action as showing that ES (pluripotent) cells "have the ability to differentiate into any cell type of any organ." As noted above, the NIH article entitled,

"Stem Cells: A Primer," teaches that pluripotent stem cells can form virtually every type of human cell.

The Examiner on page 12 of the Advisory Action again challenges Applicant's statement that totipotent cells are considered "master cells" since they have the potential to form an entire organism. Applicant's statement is believed to be consistent with the above-identified NIH article, which has not been rebutted by the Examiner. While of academic interest, the Examiner has failed to explain the nexus between the teachings of the Alberts et al. article and the disclosed and claimed invention. The conclusionary statement that, "What determines whether a cell is a neuron or a keratinocyte or a hematopoeitic cell or a totipotent cell or any cell type is the control of expression of the genome" is still not understood by Applicant. If the Examiner is relying on Alberts et al. to discredit the claimed invention by contending that it is known to use any cell type through control of expression of the genome to produce a human organism, she has not provided evidence of this. In any event, the Examiner has not explained the nexus between such bogus reasoning and the anticipation rejection in issue.

Applicant has maintained throughout the prosecution of this invention that his disclosure and claims do not include the use of totipotent cells since such cells would not provide the claimed desired therapeutic effect of morphogenetically growing a desired soft tissue at a selected site in a human patient's body. The Examiner's states on page 12 of the Advisory Action that, "The specification does not teach exclusion of totipotent cells." The relevance of such statement is not understood. That the specification does not teach using totipotent cells is also true. There is no requisite Applicant is aware of that a specification must teach things that do not work to provide the desired therapeutic effect in order to satisfy the statutory requirements of

Title 35 of the U.S. Code. There is clearly a difference in kind between the type of cells used and the achieved therapeutic result claimed by applicant and the in-vitro fertilization process taught by Lutjen.

Applicant submits that claims 382-402 define novel subject matter within the purview of 35 U.S.C §102, over the Lutjen publication and favorable reconsideration and allowance of said claims is respectfully requested.

Applicant again points out that the instant application has been pending since April 21, 1998 – a period of over eight years, is entitled to Special Status during prosecution, and is subject to term erosion. Accordingly, Applicant again requests that prosecution of this application should proceed as timely and efficiently as possible. In other words, the PTO is requested to follow the tenants of compact prosecution. To assist in concluding the instant prosecution, Applicant has provided further evidence that should conclusively resolve all outstanding issues and result in the allowance of the application. Applicant believes the Examiner should consider and interpret the disclosed and claimed terminology in a manner consistent with evidence submitted by Applicant (regarding how such terminology is understood and used by those skilled in the medical art) and then apply the established meaning of such terminology to the instant claims. The scope of the claims would then be interpreted in terms understood and used by those skilled in the medical art.

From the foregoing remarks, Applicant submits that the instant application is in condition for allowance, and a Notice to such effect is respectfully requested. Should the Examiner have any questions or require additional information or discussion to place the application in condition for allowance, a phone call to the undersigned attorney would be appreciated.

Respectfully submitted,

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Control of CREB-binding Protein Signaling by Nuclear Fibroblast Growth Factor Receptor-1

A NOVEL MECHANISM OF GENE REGULATION*

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ABSTRACT

In integrative nuclear fibroblast growth factor receptor-1 (FGFR1) signaling a newly synthesized FGFR1 translocates to the nucleus to stimulate cell differentiation and associated gene activities. The present study shows that FGFR1 accumulates and interacts with the transcriptional co-activator CREB-binding protein (CBP) in nuclear speckle domains in the developing brain and in neural progenitor-like cells *in vitro*, which accompanies differentiation and postmitotic growth. Cell differentiation and gene activation by nuclear FGFR1 do not require tyrosine kinase activity. Instead, FGFR1 stimulates transcription in cooperation with CBP by increasing recruitment of RNA polymerase II and histone acetylation at the active gene

promoter. FGFR1 is a multifactorial protein whose N terminus interacts with CBP and C terminus with ribosomal S6 kinase 1 (RSK1). Nuclear FGFR1 augments CBP-mediated transcription by 1) releasing the CBP C-terminal domain from RSK1 inhibition and 2) activating the CBP N-terminal domain. The interaction of FGFR1 with CBP and RSK1 allows activation of gene transcription and may play a role in cell differentiation.

INTRODUCTION

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During ontogeny, cells in the nervous system and in other tissues multiply, grow, and differentiate under the control of a plethora of extracellular signals. Progression through developmental phases requires concerted regulation of multiple genes, which occurs at the level of sequence specific transcription factors and at chromatin locations where remodeling occurs following histone modifications (1). Central characters in coordinating these events are transcription co-activators CREB-binding protein (CBP)[1] and its homolog p300. CBP and/or p300 function at the ends of several signaling cascades and interact with components of the basal transcription machinery. CBP/p300 have intrinsic histone acetyltransferase activity and provide scaffolding for the multiprotein complexes that facilitate formation and stabilization of the RNA pol II holoenzyme complex and chromatin remodeling essential for initiation and continued transcription (2).

CBP activity can be affected by phosphorylation with a variety of serine/threonine kinases (3, 4) as well as by methylation (5, 6). CBP functions are also influenced by cross-talk among distinct signaling cascades that compete for the limiting amounts of CBP (7–9). For instance, Ras-mediated mitogenic stimulation induces binding of CBP to serine/threonine ribosomal S6 kinase (pp90 RSK1), which phosphorylates and activates CREB and other sequence-specific transcription factors. Sequestering of CBP by RSK1 appears to channel the thrust of cell stimulation via the Ras pathway (*i.e.* mitogen activation of the c-*fos* gene) while blocking cAMP-mediated stimulation of the tyrosine hydroxylase gene (specific for differentiated catecholaminergic cells) (10). Hence, dynamic associations between CBP and RSK1 could allow CBP to activate distinct sets of genes and thereby promote different ontogenic phases. However, how the CBP-RSK interaction may be regulated by extracellular and intracellular signals is unknown.

The recently described integrative nuclear FGFR1 signaling has been shown to integrate diverse signals that regulate cell growth, differentiation, and gene transcription (11). Upon cell stimulation, a newly synthesized FGFR1, a typically plasma membrane-directed protein, is released into the cytosol by a mechanism that appears analogous to the retranslocation of endoplasmic reticulum transmembrane proteins to the cytosol by Sec61 (12). The cytoplasmic mobilization of FGFR1 is enabled by the FGFR1 transmembrane domain, whose hydrophobic chain is interrupted by polar amino acids and has a predicted β-sheet-like structure, atypical for transmembrane peptides. FGFR1 is transported into the nucleus by importin-β (13, 14), where it can interact with other proteins (15, 16). The presence of

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Epidermal Growth Factor Binds to a Receptor on *Trypanosoma cruzi* Amastigotes Inducing Signal Transduction Events and Cell Proliferation

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ABSTRACT. Host growth factors induce proliferation of *Trypanosoma cruzi* amastigotes by mechanisms that remain poorly defined. Here we examined human epidermal growth factor (EGF) for its ability to bind to the mammalian multiplicative forms of *T. cruzi* and to induce growth of the parasites. EGF stimulated incorporation of [³H] thymidine into DNA and growth of amastigotes both in a concentration-dependent manner. Radiolabeled EGF was found to bind to amastigotes in a concentration-dependent and saturable manner but it did not bind to trypomastigotes. Scatchard analysis showed a single class of receptors with a K_d of 0.8 nM and numbering 3.1 × 10^5 per amastigote. Results from internalization experiments provided evidence of receptor-mediated endocytosis of EGF. Northern analysis showed a 3.0-kb transcript for the putative EGF receptor (EGFR) homologue in amastigotes, but not trypomastigotes. Binding of EGF to amastigotes induced signal transduction events. EGF induced "in vitro" kinase activity as determined by γ-[³²P] ATP incorporation into amastigote proteins. EGF also increased protein kinase C activity in a concentration-dependent manner and Mitogen Activated Protein (MAP) kinase activity in a time- and concentration-dependent manner. A specific inhibitor (AG14782) of the EGFR and a MAP kinase inhibitor (PD98059) decreased EGF-dependent *T. cruzi* MAP kinase activity. These results describe a novel mechanism used by amastigotes to regulate their proliferation mediated by an EGF-dependent signal transduction pathway.

Key Words. Chagas' disease, EGF receptors, kinase activity, ligand-receptor binding, MAP kinase, Northern blot, protein kinase C, receptor-mediated endocytosis, Scatchard Plot, trypanosome growth.

Trypanosoma cruzi is an obligate intracellular protozoan and the causative agent of Chagas' disease. This fatal and debilitating disease affects over 100 million people in Central and South America (Kierszenbaum 1996). Once trypomastigotes enter the bloodstream, they invade a variety of host cell types, and transform into amastigotes, which multiply intracellularly before converting into the invasive trypomastigotes (Brenner 1973).

The mechanisms of amastigote replication and growth are not yet fully understood. Scarce information has been presented in the literature about amastigote biology and growth regulation. The fact that these forms of the parasite grow axenically (Villalta and Kierszenbaum 1982) has allowed us to test the hypothesis that amastigote multiplication is influenced by factors involved in the growth and differentiation of host cells. Some of these host growth factors might regulate the intracellular replication of *T. cruzi* amastigotes via signal transduction. Therefore, understanding growth factor-induced regulation in *T. cruzi* amastigotes may aid in the development of strategies aimed at halting the proliferation of these forms of the parasite.

Epidermal growth factor (EGF) is a multifactorial growth factor that activates signal transduction events in mammalian cells, induces the proliferation of many cells in vitro, and of epidermal cells in vivo (Carpenter and Whal 1991). The epidermal growth factor receptor (EGFR) is found on host cells as a 170kD transmembrane protein with intrinsic tyrosine kinase activity (Earp et al. 1995). It is present at 10^4 (fibroblasts) to 10^6 (epidermal carcinoma cells) receptors per cell (Carpenter and Whal 1991). Signal transduction events mediated via the EGFR in mammalian hosts include the Mitogen Activate Protein kinase (MAP kinase) pathway, Jak-STAT pathway, and other events in this complex network of molecule-molecule interactions (Van der Geer, Hunter, and Linberg 1994).

In this paper we report the novel observations that EGF induces growth of axenic *T. cruzi* amastigotes, that amastigotes possess but invasive trypomastigotes lack surface EGF receptors, and that upon binding of EGF to the EGF receptor on amastigotes the EGF is internalized by receptor-mediated endocytosis with associated signal-inducing transduction events.

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MATERIALS AND METHODS

Organisms. *Trypanosoma cruzi* amastigotes released from Vero cells (ATCC, grown in Minimum Essential Medium supplemented with 10% fetal bovine serum in the presence of 100 U penicillin/ml and 100 μg streptomycin) were purified by differential centrifugation on a metrizamide gradient as previously described (Lima and Villalta 1990). Pure amastigotes were then grown at 37 °C in 5% CO_2 atmosphere for 48 h in cell-free ML-15 medium (Gibco, BRL, Grand Island, New York) supplemented with 10% FBS (Lima and Villalta 1990), washed with Hanks' Balanced Salt Solution, pH 7.2 (HBSS), and starved before exposure to EGF as described below. All experiments performed in this study used axenically grown amastigotes. Trypomastigotes were obtained from the supernatants of rat heart myoblast monolayers as described (Lima and Villalta 1989). Both amastigotes and trypomastigotes were washed thoroughly with HBSS before being tested in ligand-binding assays as described below.

EGF induced ^{3}[H]-thymidine incorporation and growth of axenic amastigotes. Axenically grown amastigotes were starved for 24 h by incubating in Dulbecco's Minimum Essential Medium (Gibco BRL) supplemented with 1% endotoxin-free bovine serum albumin (BSA, Bayer, Kankakee, IL) (DMEM + BSA). Starved organisms were incubated for 24 h in different concentrations of EGF ranging from 0.1 to 40 ng/ml in RPMI medium (Gibco, BRL) containing 1 μCi of ^{3}[H]-thymidine (New England Nuclear, NEN, Boston, MA). Parasites were harvested using a PHD cell harvester and trichloroacetic (TCA) precipitable radioactivity was counted in a scintillation counter. To directly evaluate the effect of EGF on the growth of amastigotes, axenically grown and starved amastigotes were incubated with 1 ng/ml EGF in ML-15 medium for 24–96 h in the absence of serum or BSA and the number of organisms present in the cultures was determined. Controls for these experiments were performed incubating organisms in the absence of growth factor.

Binding assays. Binding of ^{125}I-labeled EGF (NEN; specific activity, 175 μCi/μg dissolved in 500 μl of milli-Q water) to amastigotes and trypomastigotes was carried out in 1.5-ml Eppendorf tubes that had been pre-coated with 10% BSA in PBS as described (Lima and Villalta 1990). Fifty microliters of amastigote or trypomastigote suspension (5 × 10^6 cells/ml in HBSS-BSA) were added to the tubes, followed by 50 μl of HBSS-BSA containing increasing concentrations of ^{125}I-labeled

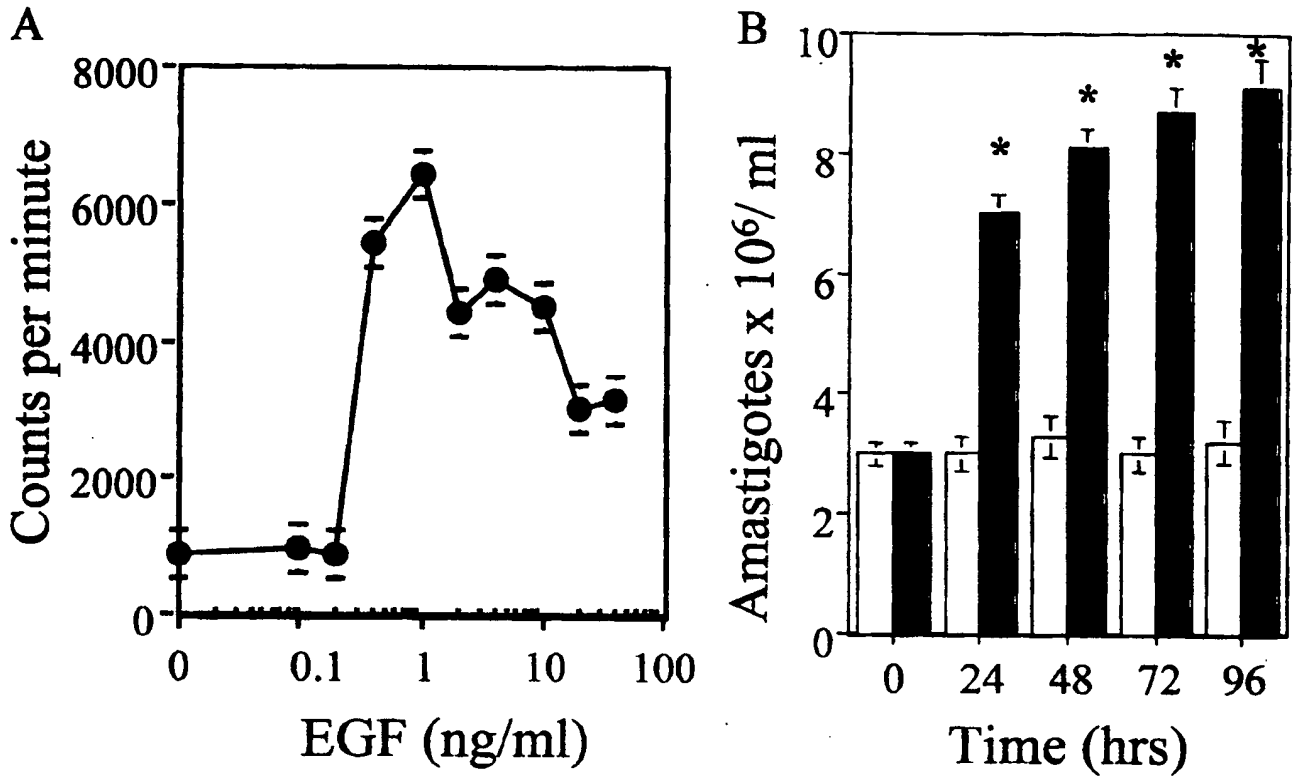

Fig. 1. Epidermal growth factor stimulates DNA synthesis and proliferation of *Trypanosoma cruzi* amastigotes. Panel A. Incorporation of [³H]-thymidine. Overnight starved amastigotes (3 × 10⁶ organisms/ml) in RPMI with 1% BSA were incubated at 37 °C with different concentrations of EGF and 1 μCi of ³[H]-thymidine. After 24 h, the cells were harvested and the incorporation of thymidine into DNA was determined by measuring acid-insoluble radioactivity. Panel B. Cell proliferation. Overnight starved amastigotes (3 × 10⁶ organisms/ml as in A) were incubated at 37 °C in ML-15 medium (open bars) or in medium containing 1 ng/ml EGF in ML-15 (closed bars). The number of organisms/ml was determined at 24-h intervals. Bars represent means (±1 S.D.) from triplicate samples. * Indicates a significant difference compared to 0 h ($p < 0.05$).

EGF, ranging from 0.18–3.0 ng ml/ml in HBSS-BSA and 50 μl of HBSS-BSA alone, or containing 100-fold molar excess of unlabeled EGF to determine non-specific binding. Each concentration was tested in triplicate. The tubes were incubated at 4 °C for 1 h with constant shaking. Unbound radiolabeled EGF was removed by three washes with HBSS by centrifugation at 4 °C and the radioactivity associated with the cellular pellet was determined in a gamma counter. Specific binding was determined by subtracting non-specific binding (determined in the presence of excess unlabeled EGF) from the total counts (Lima and Villalta 1990). The amount of EGF bound to amastigotes or trypomastigotes was calculated as pg EGF = (Bound cpm) × (Specific activity)/(cpm/μl). Scatchard analysis was performed using Prism (Graph Pad Software for Science, San Diego, CA).

Receptor-mediated endocytosis. Endocytosis of receptor-bound EGF by amastigotes was evaluated as described before (Lima and Villalta 1990). Briefly, a triplicate group of Eppendorf tubes (1.5 ml) received 50-μl portions of the amastigote suspension (2 × 10⁷ amastigotes ml/ml in HBSS) followed by 50 μl of HBSS containing 18 ng of ¹²⁵I-labeled EGF or HBSS alone for 1 h at 4 °C or 37 °C. Unbound ¹²⁵I-labeled EGF was removed by centrifugation at 4 °C. To half of the amastigote samples, 50 μl of chilled (4 °C) 0.25 M acetic acid/0.5 M NaCl was added and after 5 sec the pH was immediately adjusted to 7.0 by the addition of 1 M sodium acetate. These conditions dissociate bound EGF from its receptor on mammalian cells. The other half of the amastigote samples received 50 μl of HBSS instead of the acetic acid/NaCl for 5 sec. Cells were centrifuged at 13,000 *g* for 5 min and radioactivity associated with the pellets was determined using a gamma counter. This brief treatment of amastigotes had no noticeable effect on recoverable cell numbers, on the morphology of the cells or on their ability to grow in ML-15 medium.

Northern blot analysis. RNA extracted from amastigotes and from trypomastigotes was obtained using Trizol (Gibco, BRL) as described (Sambrook, Fritsch, and Maniatis 1989). Twenty micrograms of total RNA was subjected to electrophoresis in a 1.5% agarose gel containing 5.5% (w/v) formaldehyde, transferred to nylon membranes and tested for hybridization with a [³²P] labeled nick-translated probe (λHER41) corresponding to a 1.8-kb cDNA fragment of the human EGFR gene (ATCC) (Ullrich et al. 1984). Hybridization was performed in 2.0× SSC/0.2% SDS for 24 h at 42 °C. The membranes were then washed twice with 2× SSC/0.2% SDS for 30 min at 65 °C followed by two washes of 30 min each at 65 °C with 0.2× SSC/0.2% SDS, and the blot was developed by autoradiography. Ethidium bromide stain of RNA from trypomastigotes and amastigotes were used as loading controls for Northern blot analysis.

Southern blot analysis. Genomic DNA of *T. cruzi* was isolated as described (Sambrook, Fritsch, and Maniatis 1989).

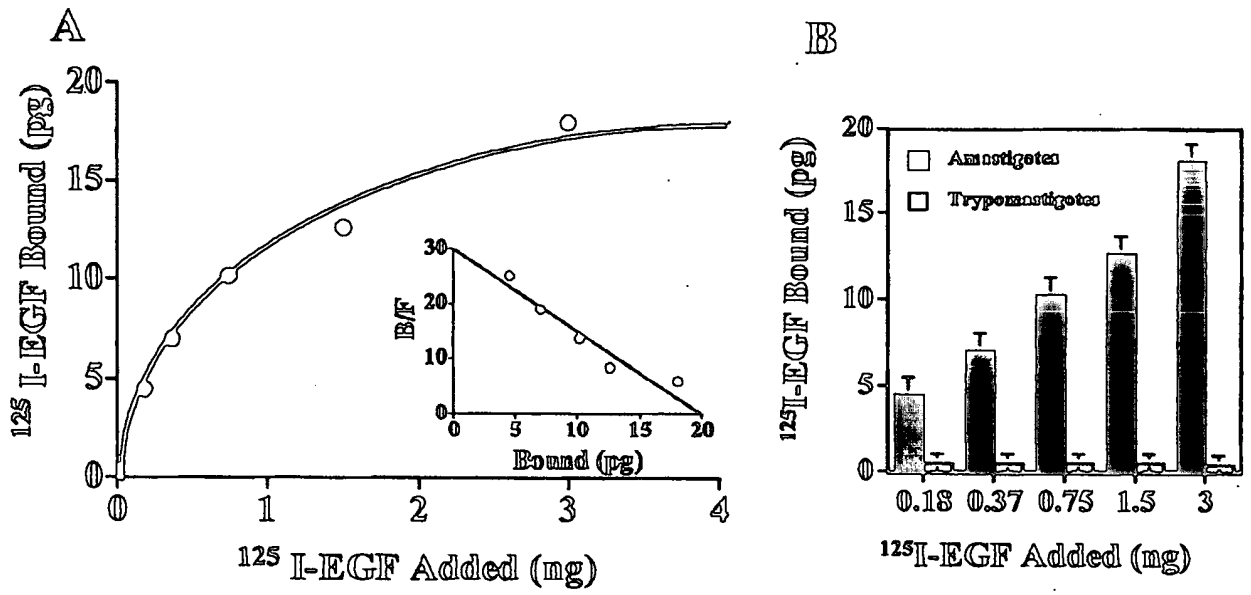

Fig. 2. Epidermal growth factor binds to amastigotes of *Trypanosoma cruzi*. Panel A. Binding of ^{125}I-labeled EGF to amastigotes was performed and assessed as described in Materials and Methods. Insert in Panel A is a Scatchard analysis of the binding data. B/F = Ratio of Bound:Free (unbound) EGF. Panel B. EGF does not bind to trypomastigotes. Comparative binding of ^{125}I-labeled EGF to amastigotes and trypomastigotes was carried out as described in Materials and Methods. Bars represent means from triplicate samples (± 1 S.D.).

DNA was digested with the restriction enzymes *EcoR*I, and *Pvu* 2 and the digests were separated on 0.8% agarose, transferred to nylon membranes and probed with [^{32}P] labeled nick-translated probe (λHER41) corresponding to a 1.8-kb cDNA fragment of the human EGFR gene (ATCC) (Ullrich et al. 1984). Hybridization was performed in 5× Denhardt's solution 0.5% SDS, 50 mg/ml salmon sperm DNA, 6× SSPE and 10% dextran sulfate at 65 °C for 24 h. The membranes were washed twice with 2× SSC/0.2% SDS for 30 min at 65 °C followed by two washes of 30 min each at 65 °C with 0.1× SSC/0.2% SDS, and the blots were developed by autoradiography.

Protein kinase assay. EGF-treated (2 ng /ml for 15 min at 4 °C) and mock-treated amastigotes were solubilized at 4 °C for 2 h in 1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) containing the protease inhibitors (1 mM phenylmethylsulfonyl fluoride, PMSF, 1 mM p-tosyl-L-lysinechloromethylketone, TLCK and 3 μg /ml aprotinin) and 100 μM of orthovanadate phosphatase inhibitor as described (Lima and Villalta 1989). Samples were centrifuged for 5 min at 50,000 *g*, and CHAPS lysates were quickly frozen in liquid nitrogen and stored at −70 °C until use. These lysates were incubated with kinase buffer (20 mM Hepes, pH 7.4, 25 mM $MgCl_2$, 4 mM $MnCl_2$, 100 μM Na_3VO_4, 10 mM NaF and 10 μg/ml BSA) at room temperature for 10 min. The reaction was initiated by the addition of 10 μCi of γ-[^{32}P]-ATP and allowed to proceed for 30 min at room temperature. The reaction was stopped by adding 3× Laemmli running buffer and boiling the sample. Samples were then analyzed by electrophoresis under non-reducing conditions on polyacrylamide gels in the presence of sodium dodecylsulfate (SDS-PAGE). Gels were dried in cellophane, exposed to X-ray film overnight and developed by autoradiography. Films were analyzed with a densitometer to quantitate differences in the level of phosphorylation.

Protein kinase C (PKC) activity. The ability of EGF to induce PKC activity in amastigotes was investigated using a non-radioactive protein-kinase assay kit (Calbiochem, La Jolla, CA) measured PKC activity in cell lysates (Villalta et al. 1999). The kit is based on an enzyme-linked immunosorbent assay that utilizes an immobilized synthetic PKC pseudo-substrate on serine, which is recognized by a biotinylated monoclonal antibody. Upon binding of peroxidase-conjugated streptavidin, the colorimetric reaction was developed with o-phenylenediamine. The component mixture of the assay included 12 μl of 10× PKC reaction buffer (250 nM Tris-HCl, pH 7.0, 30 mM $MgCl_2$, 20 mM $CaCl_2$, 5 mM EDTA, 10 mM EGTA and 50 mM β-mercaptoethanol), 12 μl of 1 mM ATP, 12 μl of 500 mg /ml phosphatidylserine (PS) and 72 μl of endotoxin-free Milli-Q water. The negative control for the assay contained an equal vol. of EGTA instead of phosphatidylserine (PS). This assay was used to determine the ability of EGF (2 ng /ml) to activate PKC in amastigotes. Briefly, *T. cruzi* amastigotes (1×10^7 cells/ml) were incubated in medium containing 0 or 20 mM EGTA and 0 or 2 ng/ml EGF for 0, 5, 15 or 30 min. The cells were sedimented, resuspended, and sonicated for 10 sec. The sonicates were centrifuged at 100,000 *g* for 60 min. at 4 °C and the cell-free supernatants were tested for PKC activity. Relative PKC activity was obtained by dividing PKC activity induced by EGF by the PKC activity of its respective mock-treated control.

Mitogen activated protein (MAP) kinase assays. MAP kinase activity was determined as described previously (Villalta et al. 1998). Amastigotes of *T. cruzi* (1×10^7 cells/ml) were incubated with 0 or 2.0 ng/ml EGF for 0, 5, 15, or 30 minutes or treated with 0, 0.2, 2 or 20 ng/ml EGF for 30 min. The parasites were washed with HEPES buffer, then sonicated in MAP kinase buffer (10 mM Tris-HCl, pH 7.4, 20 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After sonication for 10 s, the samples were centrifuged for 5 min at 14,000 *g*, and the supernatants were assayed for MAPK activity with a kit (Amersham Corp., Cleveland, OH) which measures the incorporation of γ-[^{32}P]-ATP into a synthetic peptide (KREL-

VEPLTPAGEAPNQALLR) that acts as a MAP kinase-specific substrate. The reaction was carried out with the cell lysate (1 μg of protein) in 75 mM HEPES buffer, pH 7.4, containing 1.2 mM $MgCl_2$, 2 mM substrate peptide, and 1.2 mM (1 μCi) of γ-[^{33}P]-ATP for 30 min at 30 °C. The resultant solution was applied to a phosphocellulose membrane which was washed thoroughly in 1% acetic acid and then in water. Radioactivity bound to the membrane was measured by liquid scintillation counting. The fold-increase in activity was calculated by dividing the control cpm values by the test sample cpm values. MAP kinase inhibition studies were performed by pre-treating *T. cruzi* amastigotes with 10 μM AG14782 in PBS (EGF inhibitor) (Eguchi et al. 1998) or mock-treating with PBS, prior to stimulation with 2.0 ng/ml EGF for 15 min or 50 μM of the MAP kinase inhibitor, PD98059, in PBS (Alessi et al. 1995) or mock-treating with PBS, prior to stimulation with 2.0 ng/ml EGF for 15 min. MAP kinase activation was measured as described above. The selected concentrations of AG14782 or PD98059 did not affect the morphology or in situ vibrational motion of amastigotes and on this basis they do not appear to be toxic (Villalta and Kierszenbaum 1982).

Statistical analysis and presentation of the results. Results of this research were obtained from triplicate values in each independent experiment and represent three independent experiments with identical protocols. Results are expressed as the mean ± one standard deviation. Differences were considered to be statistically significant at $p < 0.05$ as determined using Student's *t* test.

RESULTS

EGF induced concentration-dependent ^{3}[H]-thymidine incorporation into DNA and growth of amastigotes. At 1 ng/ml, EGF caused a 6.5-fold increase in ^{3}[H]-thymidine incorporation after 24-h exposure to the growth factor (Fig. 1A). EGF was also able to induce amastigote proliferation albeit at lower levels than EGF-induced ^{3}[H]-thymidine incorporation into the DNA of amastigotes (Fig. 1B).

EGF binds to amastigotes in a ligand-receptor interaction but not to trypomastigotes. To test for the presence of receptors for EGF on *T. cruzi*, we conducted ligand-receptor binding studies on amastigotes and trypomastigotes using radioiodinated EGF. The binding of ^{125}I-labeled EGF to amastigotes was concentration-dependent. Saturation occurred at 3.0 ng EGF, indicating the existence on this parasite of receptors that can bind EGF (Fig. 2A). Computerized Scatchard analysis of the binding data showed that there is one class of receptors for EGF on amastigotes estimated at 3.1×10^3 receptors per amastigote and a K_d of 0.8 nM (insert of Fig 2A). In contrast, we were not able to detect binding of radioiodinated EGF to trypomastigotes (Fig. 2B). Non-specific binding in these experiments was 21% of total binding indicating the low level of non-specific binding. Northern blots of *T. cruzi* amastigote RNA tested for hybridization with a human EGFR cDNA probe (Ullrich et al. 1984) indicated the presence of a transcript that hybridized with the human EGFR gene. RNA from trypomastigotes (which were used as controls in this experiment) did not contain any EGFR transcripts while a 3.0-kb transcript was observed in amastigotes, supporting the notion that there is an EGFR homologue gene in *T. cruzi* amastigotes (Fig 3). Results of Southern blots with the human EGFR probe showed that three bands of 1.6, 3.5 and 5.0 kb in *Eco*RI digests hybridized with the probe whereas *Pvu* 2 digests showed that 1.6, 3.0 and 4.5-kb bands hybridized with the human EGFR probe (data not shown), indicating the presence of an EGFR homologue gene in the genome of *T. cruzi* as a multicopy gene.

***T. cruzi* amastigotes internalize ^{125}I-labeled EGF.** The results of these experiments indicated that EGF was internalized after binding to the *T. cruzi* EGF receptor (Table 1). When ^{125}I-labeled EGF was incubated with amastigotes for 1 h at 4 °C (conditions in which ligand–receptor complex internalization does not occur) the bound EGF was readily dissociated from the parasite surface. However, EGF that had bound to amastigotes at 37 °C during a 1-h incubation could not be dissociated by acid treatment, indicating that the labeled EGF may have been internalized.

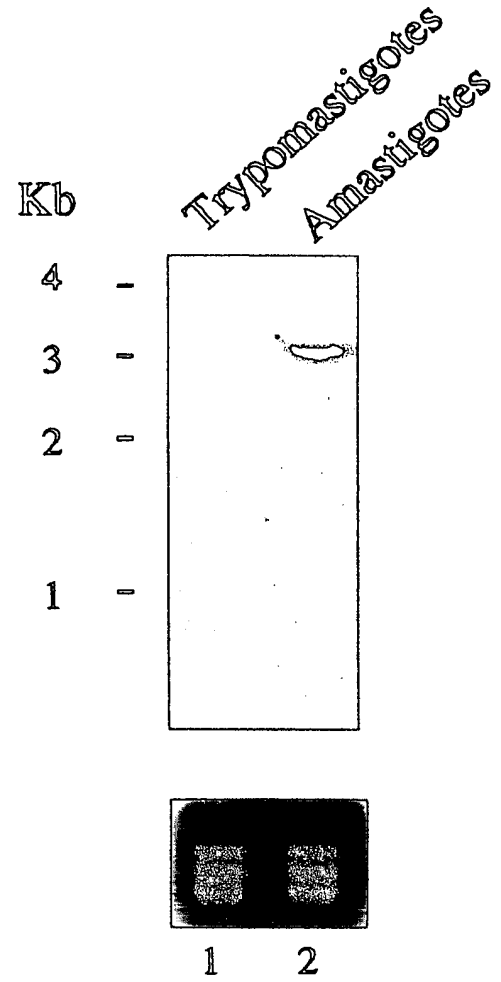

Fig. 3. Northern blot analysis of RNA from amastigotes of *Trypanosoma cruzi* present a 3.0-kb Epidermal Growth Factor Receptor transcript that hybridizes with the human Epidermal Growth Factor Receptor gene whereas trypomastigotes did not, as described in Materials and Methods. The bottom panel represents an ethidium bromide staining of the gel as RNA loading controls.

EGF-induces protein kinase activity in amastigotes. Amastigotes showed an EGF-dependent increase in in vitro kinase activity after incubation with 2.0 ng/ml EGF for 15 min. This was indicated by increased phosphorylation of a broad spectrum of proteins with molecular weights of 100 kD, 45 kD and 24 kD (Fig. 4, lane 2) as compared to mock-treated amastigotes (Fig. 4, lane 1). Scanning three autoradiographs from three independent experiments indicated that proteins at the molecular weight range of 100 kD showed an 11 ± 0.4-fold in-

Table 1. Evidence for internalization of ^{125}I-labeled EGF by amastigotes of *Trypanosoma cruzi*.

Incubation temperature	Radioactivity (cpm) in amastigotes washed in:	
	HBSS	Acid
4 °C	4096 ± 174[a]	280 ± 134
37 °C	5367 ± 983	4801 ± 947

Amastigote samples in triplicate (2×10^7/ml in HBSS) received 18 ng ^{125}I-labeled EGF or HBSS alone for 1 h at 4 °C or 37 °C. Unbound ^{125}I-labeled EGF was removed by centrifugation at 4 °C. One-half of the samples was tested for dissociation of bound EGF by treatment with acidified NaCl solution. Each point represents the mean of triplicate determinations ± 1 SD. This is a set of representative experiments of three performed with similar results.

[a] Differences between cells with and without acid treatment at 4 °C were statistically significant ($p < 0.05$).

crease in phosphorylation; proteins at the molecular weight range of 45 kD showed a phosphorylation increase of 2.3 ± 0.2-fold; and proteins at the 24 kD range showed a 1.8 ± 0.-fold increase in phosphorylation after EGF treatment.

EGF activates PKC in amastigotes. Amastigotes exposed to EGF (2.0 ng /ml) for 5, 15, and 30 min showed increased PKC activity. Activation of EGF-induced PKC activity in amastigotes could be seen as early as 5 min, and activity continued through 30 min (Fig. 5).

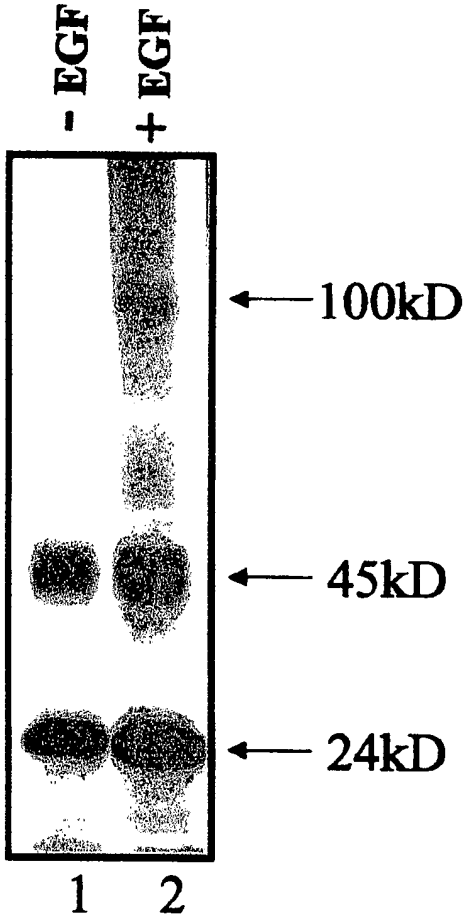

Fig. 4. Exposure of amastigotes of *Trypanosoma cruzi* to Epidermal Growth Factor induces an increase in kinase activity (Lane 2). Control was performed in the absence of growth factor (Lane 1). This is representative of three experiments.

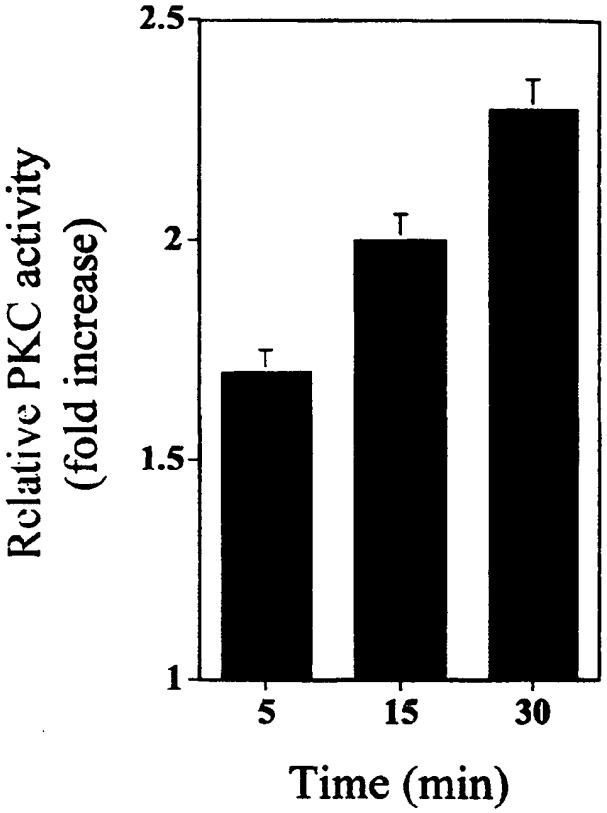

Fig. 5. Epidermal Growth Factor increases Protein Kinase C activity in amastigotes of *Trypanosoma cruzi*. Amastigotes were incubated with 2 ng/ml EGF for 5, 15 and 30 min. Controls were incubated in the absence of growth factor. PKC activity was determined as described in Materials and Methods. Each bar represents the mean of triplicate determinations ± 1 S.D. This is representative of three experiments. Differences between PKC activity in the presence and absence of EGF were statistically significant at all times ($p < 0.05$).

EGF activates MAP kinase in amastigotes. Incubation of amastigotes with 0.2–20 ng/ml EGF stimulated MAP kinase (Fig. 6A). The increase in MAP kinase activity was also time-dependent. EGF-induced MAP kinase activity in amastigotes could be seen as early as 5 min and maximal activity was seen at 15–30 min (Fig. 6B). Pre-incubation of amastigotes with 10-μM AG1478 (EGFR inhibitor) 30 min prior to stimulation with 2.0 ng/ml EGF for 15 min, significantly reduced the level of stimulation of MAP kinase by EGF (Fig. 6B). Moreover, treatment of amastigotes with 50 μM of the MAP kinase inhibitor, PD98059, for 30 min prior to stimulation with 2.0 ng/ml EGF inhibited MAP kinase activation (Fig. 6A). The selected concentrations of AG1478 and PD98059 also inhibited by 86% and 78% respectively, the subsequent stimulation of amastigote by EGF (data not shown).

DISCUSSION

This work shows for the first time that EGF is able to stimulate growth of axenic *T. cruzi* amastigotes, that EGF binds to amastigotes and that this binding induces signal transduction

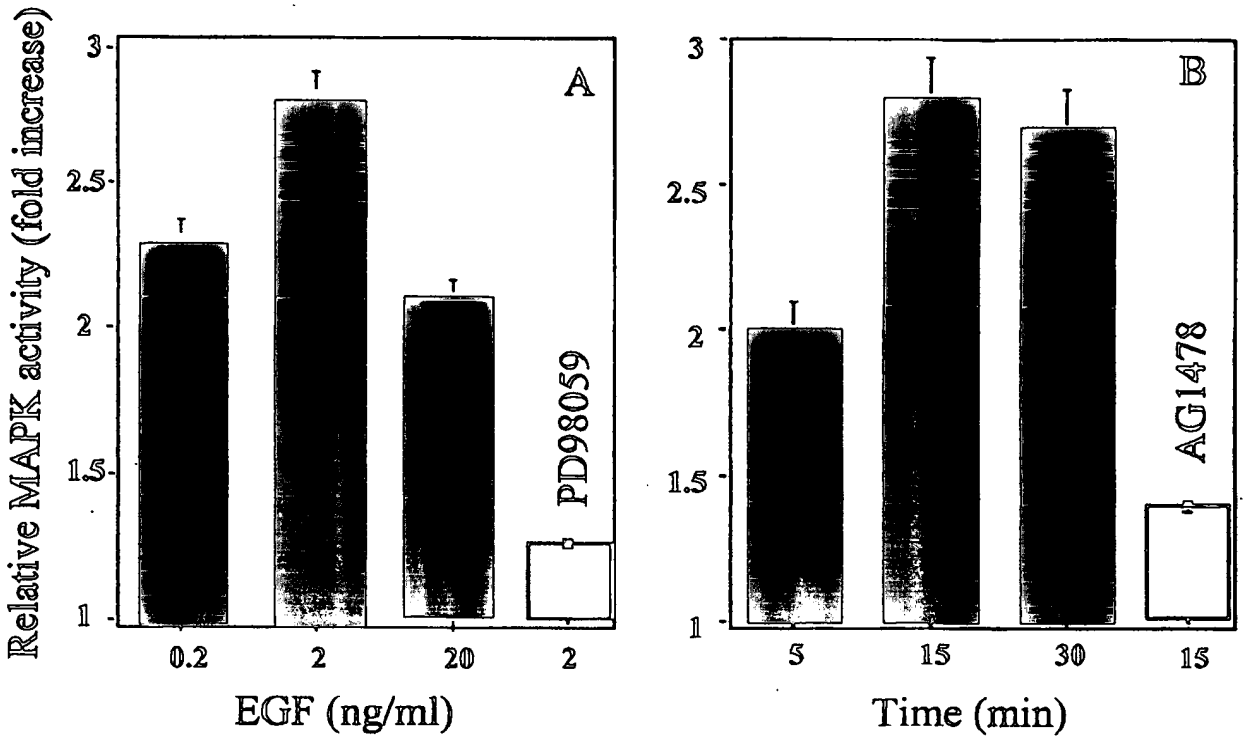

Fig. 6. Stimulation by Epidermal Growth Factor of Mitogen Activated Protein kinase activity in amastigotes of *Trypanosoma cruzi*. Panel A. Concentration-dependent activation by EGF. To test for inhibition, amastigotes were incubated with 50 μM of the MAP kinase inhibitor PD98059 or mock-treated, and then stimulated with 2.0 ng/ml EGF for 15 min. Panel B. Kinetics of MAPK activation in amastigotes induced by EGF. To test for inhibition, amastigotes were incubated with 10 μM of the EGFR inhibitor AG1478 or mock-treated, and then stimulated with 2.0 ng/ml EGF for 15 min. MAP kinase activity was determined as described in Materials and Methods. Relative MAP kinase activity was determined by dividing MAP kinase activity in the presence of EGF by the MAP kinase activity in the absence of EGF. Bars represent the means of triplicate determinations (± 1 S.D.). This is representative of three experiments. Differences between cells treated with 2 ng/ml EGF + PD98059 and cells treated with 2 ng/ml EGF were statistically significant ($p < 0.05$). Differences between cells treated with 2 ng/ml EGF + AG1478 at 15 min and cells treated with 2 ng/ml EGF at 15 min were statistically significant ($p < 0.05$).

events similar to those observed upon the binding of EGF to its receptor in mammalian cells. These results suggest that the EGF-dependent increase in growth of amastigotes is mediated by the binding of the growth factor to specific receptors on the surface of the parasites. Because of the role of EGFR in cell growth of mammalian cells (Carpenter and Whal 1991), we reasoned that EGFR homologues would be present only on amastigotes, but not on trypomastigotes since amastigotes are the intracellular replicating forms of *T. cruzi* in the mammalian host. Indeed, our results of ligand binding studies supported this notion. We have seen that EGF binds only to the multiplicative amastigotes, but not to the non-dividing but invasive trypomastigotes. The fact that a human EGFR probe hybridizes with a 3.0-kb transcript of amastigotes in Northern blots indicates that amastigotes possess a homologue of the EGFR gene. Taken together these findings strongly suggest that the mammalian multiplicative amastigote forms of *T. cruzi* use their EGFR homologue and host EGF to modulate their proliferation.

It has been reported that *T. cruzi* amastigotes possess receptors that are specific for other mammalian cell proteins, such as Fe-transferrin and lactoferrin, and that some of these receptors are involved in the transport of essential nutrients to the parasite (Lima and Kierszenbaum 1985; Lima and Villalta 1990). The amastigote EGFR would also fall in this category of essential surface receptors, since amastigotes were found to grow in the presence of EGF, and to bind EGF specifically. We do not have evidence, at this time that the amastigote EGFR only binds EGF. However, it is known that the human EGFR also binds other growth factors in the EGF family, such as Transforming Growth Factor-α and Heparin Binding-Epidermal Growth Factor among others (Carpenter and Whal 1991). Bound EGF was also probably internalized by the parasites, indicating that amastigote EGFR homologues may undergo receptor-mediated endocytosis. The flagellar pocket is an area on many trypanosomes where receptor-mediated endocytosis and exocytosis are known to occur (Clayton, Hausler, and Blattner 1995; Grab et al. 1992). It is possible that *T. cruzi* EGFR homologues are localized at the flagellar pocket of amastigotes. Currently we are attempting to determine the location of these receptors on amastigotes. Low density lipoprotein (LDL) binds to receptors on the membrane of the flagellar pocket of *T. brucei*, causing the receptors to be internalized and subsequently degraded (Coppens et al. 1988). In the case of *T. cruzi* amastigotes, transferrin binds to receptors on the parasite, which are also internalized (Lima and Villalta 1990). Internalization is important and required for EGFR activation and mediation of signal transduction events on host cells, since the receptor regulates its activity by internalization, followed by degradation or recycling (Ullrich and Schlessinger 1990). It remains to be determined how the parasite would acquire this growth factor since amastigotes in vivo are located in an intracellular location. Amastigotes could come in contact with EGF after it is endo-

cytosed by the host cell since EGF is not immediately degraded by lysosomes. It may therefore be available for binding to the intracellular amastigote (McCune and Earp 1989). Alternatively, amastigotes could secrete an EGF homologue, which would bind to the parasite EGFR to promote its growth in an autocrine manner.

One of the major events initiated by EGF binding to its receptor on mammalian cells is the transduction of intracellular signaling (Opresko et al. 1995). The presence of a putative EGFR homologue on amastigotes and EGF-mediated protein kinase, PKC and MAP kinase activation suggests that an EGF-induced signal transduction pathway is active in *T. cruzi* and also that signal transduction proteins are present in *T. cruzi*. It has been shown that EGF binds to *Trypanosoma brucei* trypomastigotes and induces growth and phosphorylation of *T. brucei* proteins (Hide et al. 1989); however, there is no further information on how signal transduction events are induced upon EGF binding to *T. brucei*. Our results indicate for the first time that protein kinase, PKC, and MAP kinase are activated after EGF binds to its receptor on *T. cruzi* amastigotes, therefore implying that PKC and MAPK are functional in *T. cruzi*. A specific EGFR inhibitor, AG1478, which prevents ATP from binding to lysine residues of the human EGFR, and the MAP kinase inhibitor, PD89059, which prevents the phosphorylation of downstream MAP kinase, inhibited MAP kinase activation. Both of these inhibitors were able to inhibit the EGF-mediated growth of amastigotes. The effect of these two inhibitors on amastigote signaling and growth by EGF suggests that the EGF-dependent *T. cruzi* MAP kinase activity is similar to mammalian host cells and that the *T. cruzi* amastigote EGFR homologues might have a similar function as the mammalian EGF receptors.

Proliferation induced by EGF plays an essential role in the growth and development of many organisms. EGFRs have been identified and sequenced in humans, mice, rats (Ullrich and Schlesinger 1990), and chickens (Lax et al. 1990). Additionally, EGFR homologues have been identified in *Drosophila* (DER) (Livneh et al. 1985), *Caenorhabditis elegans* (Let-23) (Aroian et al. 1990), *T. brucei* (Hide et al. 1989; Steinberg and McGuigan 1994), and *Schistosoma mansoni* (Ramachandran, Skelly, and Shoemaker 1996). The novel results from our study show that *T. cruzi* amastigotes present an EGFR homologue that is involved in the growth of the parasite, and that binds EGF and activates the MAP kinase signal transduction pathway. The EGFR signaling pathway thus appears to be conserved across several eukaryotic phyla. This novel information will help advance our basic knowledge of growth regulation and signal transduction pathways involved in the growth and differentiation of *T. cruzi* and ultimately, could be a potential target for chemotherapeutic intervention in Chagas' disease.

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LITERATURE CITED

Alessi, D. R., Cuenda, A., Cohen, P., Duddley, D. T. & Saltiel, A. R. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J. Biol. Chem.*, 46: 27489–27494.

Aroian, R., Koga, V. M., Menel, J. E., Ohishima, Y. & Sternberg, P. W. 1990. The Let-23 gene necessary for *C. elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature*, 348:693–699.

Brenner, Z. 1973. Biology of *Trypanosoma cruzi*. *Annu. Rev. Microbiol.*, 27:347–382.

Carpenter, G. & Whal, M. I. 1991. The epidermal growth factor. *In*: Sporn, M. B. & Roberts, A. B. (ed.), Peptide Growth Factors and their Receptors. Springer-Verlag, New York. p. 69–81.

Clayton, C., Hausler, T. & Blattner, J. 1995. Protein trafficking in kinetoplastid protozoa. *Microbiol. Rev.*, 59:325–344.

Coppens, I., Baudhuin, P., Opperdoes, F. R. & Courtoy, P. J. 1988. Receptors for the host low density lipoproteins on the hemoflagellate *Trypanosoma brucei*: purification and involvement in the growth parasite. *Proc. Natl. Acad. Sci. USA.*, 85:6753–6757.

Earp, H. S., Dawson, T. L., Li, X. & Yu, H. 1995. Heterodimerization and functional interaction between EGF receptor family members. A new signaling paradigm with implications for breast cancer research. *Breast Can. Res. Treat.*, 35:115–132.

Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, E. D., Kawakatsu, H., Owada, K. M., Hirta, Y., Marumo, F. & Inagami, T. 1998. Calcium-dependent epidermal growth factor receptor transactivator II-induced mitogen-activated in vascular smooth muscle. *J. Biol. Chem.*, 273:8890–8896.

Grab, D. J., Wells, C. W., Shaw, M. K., Webster, P. & Russo, D. C. 1992. Endocytosed transferrin in African trypanosomes is delivered to lysosomes and may not be recycled. *Eur. J. Cell Biol.*, 59:398–404.

Hide, G., Gray, A., Harrison, C. M. & Tait, A. 1989. Identification of an epidermal growth factor receptor homologue in trypanosomes. *Mol. Biochem. Parasitol.*, 36:51–59.

Kierszenbaum, F. 1996. Can a killer be arrested? *Nature Med.*, 2:10.

Lax, I., Fischer, R., Ng, C., Segre, J., Ullrich, A., Givol, D. & Schlessinger, J. 1990. Non-contiguous regions in the extracellular domain of EGF receptor define ligand-binding specificity. *Cell Regul.*, 2:337–245.

Lima, M. F. & Kierszenbaum, F. 1985. Lactoferrin effects on phagocytic cell function. Increased uptake and killing of an intracellular parasite by murine macrophage and human monocytes. *J. Immunol.*, 134:4179–4183.

Lima, M. F. & Villalta, F. 1989. *Trypanosoma cruzi* trypomastigote clones differentially express a parasite adhesion molecule. *Mol. Biochem. Parasitol.*, 33:159–170.

Lima, M. F. & Villalta, F. 1990. *Trypanosoma cruzi* receptors for human transferrin and their role. *Mol. Biochem. Parasitol.*, 8:245–252.

Livneh, E., Glazer, L., Segal, D., Schlessinger, J. & Shilo, B. Z. 1985. The *Drosophila* EGF receptor gene homologue: conservation of both hormone binding and kinase domains. *Cell*, 40:599–607.

McCune, B. K. & Earp, H. S. 1989. The epidermal growth factor receptor tyrosine kinase in liver epithelial cells. The effect of ligand-dependent changes in cellular location. *J. Biol. Chem.*, 15:15501–15507.

Opresko, L. K., Chang, C. P., Will, V. H., Burke, P. M., Gill, G. N. & Wiley, H. S. 1995. Endocytosis and lysosomal targeting of epidermal growth factor receptors are mediated by distinct sequences independent of the tyrosine kinase domain. *J. Biol. Chem.*, 270:4325–4333.

Ramachandran, H., Skelly, P. J. & Shoemaker, B. C. 1996. The *Schistosoma mansoni* epidermal growth factor receptor homologue, SER, has tyrosine kinase activity and is localized in adult muscle. *Mol. Biochem. Parasitol.*, 83:1–10.

Sambrook, J. E., Fritsch, E. F. & Maniatis, T. Molecular cloning: a laboratory manual, 2nd ed. 1989. Cold Spring Harbor Press, Plainview, N.Y.

Steinberg, J. M. & McGuigan, F. 1994. *Trypanosoma brucei*: mammalian epidermal growth factor promotes the growth of the African trypanosome bloodstream form. *Exp. Parasitol.*, 78:329–339.

Ullrich, A. & Schlessinger, J. 1990. Signal transduction with tyrosine kinase activity. *Cell*, 61:203–212.

Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Lieberman, T. A., Schlessinger, J., Downward, J., Mayes, E. L., Wittle, N., Waterfield, M. D. & Seeburg, P. H. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 cells. *Nature*, 309:418–425.

Van der Geer, P., Hunter, T. & Linberg, R. A. 1994. Receptor protein-tyrosine kinases and other signal transduction pathway. *Annu. Rev. Cell. Biol.*, 10:251–337.

Villalta, F. & Kierszenbaum, F. 1982. Growth of isolated amastigotes of *Trypanosoma cruzi* in cell-free medium. *J. Protozool.*, 29:570–576.

Villalta, F., Zhang, Y., Bibb, K. E., Burns, Jr. J. & Lima, M. F. 1998. Signal transduction in human macrophages by gp83 ligand of *Trypanosoma cruzi*: Trypomastigote gp83 ligand up-regulates trypanosome entry through the MAP kinase pathway. *Biochem. Biophys. Res. Commun.*, 249:247–252.

Villalta, F., Zhang, Y., Bibb, K. E., Pratap, S., Burns, Jr. J. & Lima, M. F. 1999. Signal transduction in human macrophages by gp83 ligand of *Trypanosoma cruzi*: Trypomastigote gp83 ligand up-regulates trypanosome entry through protein kinase C activation. *Mol. Cell Biol. Res. Commun.*, 2:64–70.

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